

We claim:

1. A clostridial toxin substrate, comprising:
- (a) a donor fluorophore;
  - (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and
  - (c) a clostridial toxin recognition sequence comprising a cleavage site,
- wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.
2. The substrate of claim 1, comprising a botulinum toxin recognition sequence.
3. The substrate of claim 2, provided that said botulinum toxin recognition sequence is not a botulinum toxin serotype B (BoNT/B) recognition sequence.
4. A botulinum toxin serotype A (BoNT/A) substrate, comprising:
- (a) a donor fluorophore;
  - (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and
  - (c) a BoNT/A recognition sequence comprising a cleavage site,
- wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

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5. The substrate of claim 4, comprising at least six consecutive residues of SNAP-25, said six consecutive residues comprising Gln-Arg, or a peptidomimetic thereof.

5 6. The substrate of claim 5, comprising at least six consecutive residues of human SNAP-25, said six consecutive residues comprising Gln<sub>197</sub>-Arg<sub>198</sub>, or a peptidomimetic thereof.

10 7. The substrate of claim 6, comprising the amino acid sequence Glu-Ala-Asn-Gln-Arg-Ala-Thr-Lys (SEQ ID NO: 1), or a peptidomimetic thereof.

8. The substrate of claim 6, comprising residues 187 to 203 of human SNAP-25 (SEQ ID NO: 2), or a peptidomimetic thereof.

15 9. A botulinum toxin serotype B (BoNT/B) substrate, comprising:  
    (a) a donor fluorophore;  
    (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said  
20 donor fluorophore; and  
    (c) a BoNT/B recognition sequence comprising a cleavage site,  
    wherein said cleavage site intervenes between  
said donor fluorophore and said acceptor and wherein,  
25 under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

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10. The substrate of claim 9, comprising at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Phe, or a peptidomimetic thereof.

5           11. The substrate of claim 10, comprising at least six consecutive residues of human VAMP-2, said six consecutive residues comprising Gln<sub>76</sub>-Phe<sub>77</sub>, or a peptidomimetic thereof.

10           12. The substrate of claim 11, comprising the amino acid sequence Gly-Ala-Ser-Gln-Phe-Glu-Thr-Ser (SEQ ID NO: 3), or a peptidomimetic thereof.

13. The substrate of claim 11, comprising an amino acid sequence selected from the group consisting of:

15           residues 55 to 94 of human VAMP-2 (SEQ ID NO: 4), or a peptidomimetic thereof;

            residues 60 to 94 of human VAMP-2 (SEQ ID NO: 4), or a peptidomimetic thereof; and

20           residues 60 to 88 of human VAMP-2 (SEQ ID NO: 4), or a peptidomimetic thereof.

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14. A botulinum toxin serotype C1 (BoNT/C1) substrate, comprising:

- 5 (a) a donor fluorophore;  
(b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and  
(c) a BoNT/C1 recognition sequence comprising a cleavage site,

10 wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

15 15. The substrate of claim 14, comprising at least six consecutive residues of syntaxin, said six consecutive residues comprising Lys-Ala, or a peptidomimetic thereof.

20 16. The substrate of claim 15, comprising at least six consecutive residues of human syntaxin, said six consecutive residues comprising Lys<sub>253</sub>-Ala<sub>254</sub>, or a peptidomimetic thereof.

17. The substrate of claim 16, comprising the amino acid sequence Asp-Thr-Lys-Lys-Ala-Val-Lys-Tyr (SEQ ID NO: 5), or a peptidomimetic thereof.

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18. The substrate of claim 14, comprising at least six consecutive residues of SNAP-25, said six consecutive residues comprising Arg-Ala, or a peptidomimetic thereof.

5           19. The substrate of claim 18, comprising at least six consecutive residues of human SNAP-25, said six consecutive residues comprising Arg<sub>198</sub>-Ala<sub>199</sub>, or a peptidomimetic thereof.

10           20. The substrate of claim 19, comprising residues 93 to 202 of human SNAP-25 (SEQ ID NO: 2), or a peptidomimetic thereof.

21. A botulinum toxin serotype D (BoNT/D) substrate, comprising:

- 15           (a) a donor fluorophore;  
            (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and  
            (c) a BoNT/D recognition sequence comprising a cleavage site,

20           wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

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22. The substrate of claim 21, comprising at least six consecutive residues of VAMP, said six consecutive residues comprising Lys-Leu, or a peptidomimetic thereof.

5 23. The substrate of claim 22, comprising at least six consecutive residues of human VAMP, said six consecutive residues comprising Lys<sub>59</sub>-Leu<sub>60</sub>, or a peptidomimetic thereof.

10 24. The substrate of claim 23, comprising the amino acid sequence Arg-Asp-Gln-Lys-Leu-Ser-Glu-Leu (SEQ ID NO: 6), or a peptidomimetic thereof.

25. The substrate of claim 22, comprising residues 27 to 116 of rat VAMP-2 (SEQ ID NO: 7), or a peptidomimetic thereof.

15 26. A botulinum toxin serotype E (BoNT/E) substrate, comprising:  
(a) a donor fluorophore;  
(b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and  
20 (c) a BoNT/E recognition sequence comprising a cleavage site,

wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein,  
25 under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

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27. The substrate of claim 26, comprising at least six consecutive residues of SNAP-25, said six consecutive residues comprising Arg-Ile, or a peptidomimetic thereof.

5           28. The substrate of claim 27, comprising at least six consecutive residues of human SNAP-25, said six consecutive residues comprising Arg<sub>180</sub>-Ile<sub>181</sub>, or a peptidomimetic thereof.

10           29. The substrate of claim 28, comprising the amino acid sequence Gln-Ile-Asp-Arg-Ile-Met-Glu-Lys (SEQ ID NO: 8), or a peptidomimetic thereof.

30. The substrate of claim 28, comprising residues 156 to 186 of human SNAP-25 (SEQ ID NO: 2), or a peptidomimetic thereof.

15           31. A botulinum toxin serotype F (BoNT/F) substrate, comprising:

- (a) a donor fluorophore;
- (b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and
- 20           (c) a BoNT/F recognition sequence comprising a cleavage site,

          wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein,  
25           under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

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32. The substrate of claim 31, comprising at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Lys, or a peptidomimetic thereof.

5 33. The substrate of claim 32, comprising at least six consecutive residues of human VAMP, said six consecutive residues comprising Gln<sub>58</sub>-Lys<sub>59</sub>, or a peptidomimetic thereof.

10 34. The substrate of claim 33, comprising the amino acid sequence Glu-Arg-Asp-Gln-Lys-Leu-Ser-Glu (SEQ ID NO: 9), or a peptidomimetic thereof.

35. The substrate of claim 31, comprising residues 27 to 116 of rat VAMP-2 (SEQ ID NO: 7), or a peptidomimetic thereof.

15 36. A botulinum toxin serotype G (BoNT/G) substrate, comprising:  
(a) a donor fluorophore;  
(b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and  
20 (c) a BoNT/G recognition sequence comprising a cleavage site,

wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein,  
25 under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

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37. The substrate of claim 36, comprising at least six consecutive residues of VAMP, said six consecutive residues comprising Ala-Ala, or a peptidomimetic thereof.

5 38. The substrate of claim 37, comprising at least six consecutive residues of human VAMP, said six consecutive residues comprising Ala<sub>83</sub>-Ala<sub>84</sub>, or a peptidomimetic thereof.

10 39. The substrate of claim 38, comprising the amino acid sequence Glu-Thr-Ser-Ala-Ala-Lys-Leu-Lys (SEQ ID NO: 10), or a peptidomimetic thereof.

40. A tetanus toxin (TeNT) substrate, comprising:

- 15 (a) a donor fluorophore;  
(b) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and  
(c) a TeNT recognition sequence comprising a cleavage site,

20 wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor.

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41. The substrate of claim 40, comprising at least six consecutive residues of VAMP, said six consecutive residues comprising Gln-Phe, or a peptidomimetic thereof.

5 42. The substrate of claim 41, comprising at least six consecutive residues of human VAMP-2, said six consecutive residues comprising Gln<sub>76</sub>-Phe<sub>77</sub>, or a peptidomimetic thereof.

10 43. The substrate of claim 42, comprising the amino acid sequence Gly-Ala-Ser-Gln-Phe-Glu-Thr-Ser (SEQ ID NO: 11), or a peptidomimetic thereof.

15 44. The substrate of claim 41, comprising an amino acid sequence selected from the group consisting of residues 33 to 94 of human VAMP-2 (SEQ ID NO: 4), or a peptidomimetic thereof;  
residues 25 to 93 of human VAMP-2 (SEQ ID NO: 4), or a peptidomimetic thereof; and  
residues 27 to 116 of rat VAMP-2 (SEQ ID NO: 7), or a peptidomimetic thereof.

20 45. The substrate of any of claims 1, 2, 3, 4, 9, 14, 21, 26, 31, 36 or 40, wherein said substrate can be cleaved with an activity of at least 1 nanomoles/minute/milligram toxin.

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46. The substrate of any of claims 1, 2, 3, 4, 9, 14, 21, 26, 31, 36 or 40, wherein said substrate can be cleaved with an activity of at least 20 nanomoles/minute/milligram toxin.

5 47. The substrate of any of claims 1, 2, 3, 4, 9, 14, 21, 26, 31, 36 or 40, wherein said substrate can be cleaved with an activity of at least 50 nanomoles/minute/milligram toxin.

10 48. The substrate of any of claims 1, 2, 3, 4, 9, 14, 21, 26, 31, 36 or 40, wherein said substrate can be cleaved with an activity of at least 100 nanomoles/minute/milligram toxin.

15 49. The substrate of any of claims 1, 2, 3, 4, 9, 14, 21, 26, 31, 36 or 40, wherein said substrate can be cleaved with an activity of at least 150 nanomoles/minute/milligram toxin.

50. The substrate of claim 1, wherein said acceptor is an acceptor fluorophore.

20 51. The substrate of claim 50, wherein said acceptor fluorophore has a fluorescent lifetime of at least 1 microsecond.

52. The substrate of claim 1, wherein said acceptor is non-fluorescent.

25 53. The substrate of claim 1, wherein said donor fluorophore is fluorescein.

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54. The substrate of claim 1, wherein said donor fluorophore is Alexa Fluor<sup>®</sup> 488.

55. The substrate of claim 1, wherein said donor fluorophore is DABCYL.

5 56. The substrate of claim 1, wherein said donor fluorophore is BODIPY.

57. The substrate of claim 1, claim 53, or claim 54, wherein said acceptor is tetramethylrhodamine.

10 58. The substrate of claim 1 or claim 55, wherein said acceptor is EDANS.

59. The substrate of claim 1, claim 53 or claim 54, wherein said acceptor is QSY<sup>®</sup> 7.

15 60. The substrate of claim 1, which is a peptide or peptidomimetic having at most 100 residues.

61. The substrate of claim 60, which is a peptide or peptidomimetic having at most 50 residues.

20 62. The substrate of claim 61, which is a peptide or peptidomimetic having at most 40 residues.

63. The substrate of claim 62, which is a peptide or peptidomimetic having at most 20 residues.

25 64. The substrate of claim 1, wherein said donor fluorophore and said acceptor fluorophore are separated by at most fifteen residues.

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65. The substrate of claim 64, wherein said donor fluorophore and said acceptor fluorophore are separated by at most ten residues.

66. The substrate of claim 65, wherein said  
5 donor fluorophore and said acceptor fluorophore are separated by at most eight residues.

67. The substrate of claim 66, wherein said donor fluorophore and said acceptor fluorophore are separated by at most six residues.

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68. A method of determining clostridial toxin protease activity, comprising the steps of:

- 5 (a) treating a sample, under conditions suitable for clostridial toxin protease activity, with a clostridial toxin substrate comprising
- (i) a donor fluorophore;
- (ii) an acceptor having an absorbance spectrum overlapping the emission spectrum of said donor fluorophore; and
- 10 (iii) a clostridial toxin recognition sequence comprising a cleavage site,

wherein said cleavage site intervenes between said donor fluorophore and said acceptor and wherein, under the appropriate conditions, resonance energy transfer is

15 exhibited between said donor fluorophore and said acceptor;

- (b) exciting said donor fluorophore; and
- (c) determining resonance energy transfer of said treated substrate relative to a control
- 20 substrate,

wherein a difference in resonance energy transfer of said treated substrate as compared to said control substrate is indicative of clostridial toxin protease activity.

69. The method of claim 68, wherein said

25 clostridial toxin substrate is a botulinum toxin substrate.

70. The method of claim 69, wherein said botulinum toxin substrate is a BoNT/A substrate comprising a BoNT/A recognition sequence.

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71. The method of claim 69, wherein said botulinum toxin substrate is a BoNT/B substrate comprising a BoNT/B recognition sequence.

72. The method of claim 69, wherein said  
5 botulinum toxin substrate is a BoNT/C1 substrate comprising a BoNT/C1 recognition sequence.

73. The method of claim 69, wherein said botulinum toxin substrate is a BoNT/D substrate comprising a BoNT/D recognition sequence.

10 74. The method of claim 69, wherein said botulinum toxin substrate is a BoNT/E substrate comprising a BoNT/E recognition sequence.

75. The method of claim 69, wherein said  
15 botulinum toxin substrate is a BoNT/F substrate comprising a BoNT/F recognition sequence.

76. The method of claim 69, wherein said botulinum toxin substrate is a BoNT/G substrate comprising a BoNT/G recognition sequence.

77. The method of claim 68, wherein said  
20 clostridial toxin substrate is a TeNT toxin substrate comprising a TeNT recognition sequence.

78. The method of claim 68, wherein said sample is a crude cell lysate.

79. The method of claim 68 or 70 to 77, wherein  
25 said sample is isolated clostridial toxin.

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80. The method of claim 68 or 70 to 77, wherein said sample is isolated clostridial toxin light chain.

81. The method of claim 68, wherein said sample is a formulated clostridial toxin product.

5 82. The method of claim 68, wherein said sample is BOTOX<sup>®</sup>.

83. The method of claim 68, step (c) comprising detecting donor fluorescence intensity of said treated substrate,

10 wherein increased donor fluorescence intensity of said treated substrate as compared to said control substrate is indicative of clostridial toxin protease activity.

84. The method of claim 68, step (c) comprising detecting acceptor fluorescence intensity of said treated substrate,

15 wherein decreased acceptor fluorescence intensity of said treated substrate as compared to said control substrate is indicative of clostridial toxin protease  
20 activity.

85. The method of claim 68, step (c) comprising detecting an acceptor emission maximum and a donor fluorophore emission maximum of said treated substrate,

25 wherein a shift in emission maxima from near said acceptor emission maximum to near said donor fluorophore emission maximum is indicative of clostridial toxin protease activity.

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86. The method of claim 68, step (c) comprising detecting the ratio of fluorescence amplitudes near an acceptor emission maximum to the fluorescence amplitudes near a donor fluorophore emission maximum,

5 wherein a decreased ratio in said treated sample as compared to the control sample is indicative of clostridial toxin protease activity.

87. The method of claim 68, step (c) comprising detecting the excited state lifetime of the donor  
10 fluorophore in said treated substrate,  
wherein an increased donor fluorophore excited state lifetime in said treated substrate as compared to said control substrate is indicative of clostridial toxin protease activity.

15 88. The method of claim 68, further comprising repeating step (c) at one or more later time intervals.

89. The method of claim 68, wherein at least 90% of said clostridial toxin substrate is cleaved.

20 90. The method of claim 68, wherein at most 25% of said clostridial toxin substrate is cleaved.

91. The method of claim 90, wherein at most 15% of said clostridial toxin substrate is cleaved.

92. The method of claim 91, wherein at most 5% of said clostridial toxin substrate is cleaved.

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93. The method of claim 68, wherein the conditions suitable for clostridial toxin protease activity are selected such that the assay is linear.

5           94. The method of claim 68, wherein said acceptor is an acceptor fluorophore.

95. The method of claim 68, wherein said acceptor is non-fluorescent.